# Lipid composition of the stratum corneum and cutaneous water loss in birds along an aridity gradient

Champagne, Alex M.; Munoz-Garcia, Agusti; Shtayyeh, Tamer; Tieleman, B. Irene; Hegemann, Arne; Clement, Michelle E.; Williams, Joseph B.

*Published in:*
Journal of Experimental Biology

*DOI:*
10.1242/jeb.077016



*Publication date:*
2012

The Journal of Experimental Biology 215, 4299-4307

doi:10.1242/jeb.077016

## RESEARCH ARTICLE

# Lipid composition of the stratum corneum and cutaneous water loss in birds along an aridity gradient

Alex M. Champagne[1,*], Agustí Muñoz-Garcia[1], Tamer Shtayyeh[1], B. Irene Tieleman[2], Arne Hegemann[2], Michelle E. Clement[1] and Joseph B. Williams[1]

[1]Department of Evolution, Ecology, and Organismal Biology, Ohio State University, Aronoff Laboratory, 318 W. 12th Avenue, Columbus, OH 43210, USA and [2]Animal Ecology Group (CEES), University of Groningen, Centre for Life Sciences, P.O. Box 11103, 9700 CC Groningen, The Netherlands

*Author for correspondence (champagne.7@osu.edu)

## SUMMARY

**Intercellular and covalently bound lipids within the stratum corneum (SC), the outermost layer of the epidermis, are the primary barrier to cutaneous water loss (CWL) in birds. We compared CWL and intercellular SC lipid composition in 20 species of birds from desert and mesic environments. Furthermore, we compared covalently bound lipids with CWL and intercellular lipids in the lark family (Alaudidae). We found that CWL increases in birds from more mesic environments, and this increase was related to changes in intercellular SC lipid composition. The most consistent pattern that emerged was a decrease in the relative amount of cerebrosides as CWL increased, a pattern that is counterintuitive based on studies of mammals with Gaucher disease. Although covalently bound lipids in larks did not correlate with CWL, we found that covalently bound cerebrosides correlated positively with intercellular cerebrosides and intercellular cholesterol ester, and intercellular cerebrosides correlated positively with covalently bound free fatty acids. Our results led us to propose a new model for the organization of lipids in the avian SC, in which the sugar moieties of cerebrosides lie outside of intercellular lipid layers, where they may interdigitate with adjacent intercellular cerebrosides or with covalently bound cerebrosides.**

Key words: cerebroside, desert, mesic, covalently bound lipid.

Received 2 July 2012; Accepted 29 August 2012

## INTRODUCTION

Among endotherms, birds have the highest mass-specific rates of total evaporative water loss (Williams and Tieleman, 2005), accounting for five times the water lost through urine and feces in many small bird species (Bartholomew, 1972; Dawson, 1982; Williams and Tieleman, 2000). Because cutaneous water loss (CWL) accounts for approximately 65% of total evaporative water loss (Tieleman and Williams, 2002; Ro and Williams, 2010), one can imagine that selection has modified the avian integument to minimize CWL, especially for species living in dry environments.

The outermost layer of the avian integument, the stratum corneum (SC), is composed of flat, dead cells called corneocytes embedded within a lipid matrix (Bouwstra, 1997). This lipid matrix is the primary barrier to water vapor diffusion from the animal to the environment (Menon et al., 1992; Simonetti et al., 1995; Meuwissen et al., 1998). The composition and arrangement of the lipids within this matrix appears to be crucial in determining the rate of CWL.

The lipids of the avian SC consist primarily of cerebrosides, ceramides, cholesterol, free fatty acids, triacylglycerides, fatty acid methyl esters and cholesterol esters (Menon et al., 1986; Haugen et al., 2003; Muñoz-Garcia et al., 2006; Ro and Williams, 2010). These lipids may be divided into two categories based on their arrangement within the SC, covalently bound lipids (CBLs) and intercellular lipids. The CBLs covalently bind to glutamate residues of the protein involucrin on the corneocyte surface at the ω end of each fatty acid chain (Swartzendruber et al., 1987; Wertz and Downing, 1987; Wertz et al., 1989; Downing, 1992; Stewart and Downing, 2001). Although the exact mechanism is unknown, CBLs are thought to orchestrate the structure of the intercellular lipids, which are organized into layers called lamellae (Wertz, 2005; Koch et al., 2005; Gu et al., 2008). Current models of the SC, mostly constructed for the skin of mammals, envision that lipid molecules within lamellae of the SC interact to form bilayers (Wertz and Downing, 1982) or arrange in trilayers to form sandwich-like structures (Bouwstra et al., 2000).

A striking difference between mammalian and avian SC is the presence of cerebrosides in the latter (Muñoz-Garcia and Williams, 2005; Gu et al., 2008). Cerebrosides are ceramides with a hexose moiety attached to the headgroup. In mammalian SC, the enzyme β-glucocerebrosidase cleaves the sugar group from cerebrosides before they reach the SC to convert them to ceramides. In mammals, a defect in this enzyme causes a pathological condition called Gaucher disease that results in scaly skin and high rates of CWL (Holleran et al., 1994). CWL may increase in patients with Gaucher disease because the bulky sugar moieties of the cerebrosides disrupt lipid packing and the hydroxyl groups of these sugars bind with water to increase SC hydration and, therefore, permeability (Rawlings and Matts, 2005). In birds, β-glucocerebrosidase converts some, but not all, cerebrosides to ceramides (Cox et al., 2008), resulting in cerebrosides making up a substantial portion of the lipids in the SC (Muñoz-Garcia and Williams, 2005; Gu et al., 2008). However, despite the fact that birds have higher rates of CWL than do mammals, this does not seem to

be a result of cerebroside content. In several studies, increases in cerebroside content in the SC were associated with decreases in rates of CWL. House sparrows (*Passer domesticus*) from the desert of Saudi Arabia exhibit rates of CWL 25% lower than conspecifics from mesic Ohio, and these desert house sparrows have higher percentages of both intercellular and covalently bound cerebrosides than house sparrows from Ohio (Gu et al., 2008; Muñoz-Garcia and Williams, 2005). Desert birds also tend to have a greater proportion of more polar ceramides with longer fatty acid tails, in agreement with mammalian models (Shaefer and Redelmeier, 1996; Haugen et al., 2003; Lillywhite, 2006; Muñoz-Garcia et al., 2008).

The correlation between low rates of CWL and higher amounts of cerebrosides in birds suggests a fundamental difference in the organization of intercellular lipids and CBLs between mammals and birds. Furthermore, this correlation suggests that the arrangement of lipid classes, and the manner in which they interact, is more important than the composition of lipid classes *per se*. Models of the arrangement of lipids in the SC are valuable tools in predicting the roles of certain lipid classes in regulating CWL. In current models, intercellular lipids in avian SC are organized in trilayers (Muñoz-Garcia et al., 2005), and are separated from CBLs by a layer of water (Gu et al., 2008; Clement et al., 2012). However, these models are based only on data from house sparrows. Studies across a number of bird species from multiple environments may help us understand how SC lipids are organized in birds relative to CWL. Haugen et al. (Haugen et al., 2003) compared intercellular lipid composition in eight species of larks (Alaudidae) across an aridity gradient, but did not test for cerebrosides, and Ro and Williams (Ro and Williams, 2010) compared intercellular lipid composition in 12 species of birds from a mesic environment. Neither of these studies included CBLs in their analysis.

In the present study, we correlate CWL with environment and intercellular lipid composition in 20 species of birds from both mesic and arid environments. Furthermore, we correlate CBLs with environment and CWL for seven species of larks (Alaudidae) across disparate environments. Finally, we make comparisons between intercellular lipids and CBLs in larks to make inferences about the potential interactions between intercellular lipids and CBLs. Our results offer compelling evidence that an increase in cerebroside content in the avian SC is associated with a decrease in CWL, and that intercellular lipids and CBLs interact in concert to form a barrier to water loss. Our findings prompted us to proffer a new model for lipid organization in the avian SC.

## MATERIALS AND METHODS

### Capture of birds

We captured larks with mist nets at several locations along an aridity gradient. Hoopoe larks [*Alaemon alaudipes* (Desfontaines 1789)], Dunn's larks [*Eremalauda dunni* (Shelley 1904)], desert larks (*Ammomanes deserti* Lichtenstein 1823), black-crowned finchlarks [*Eremopterix nigriceps* (Gould 1841)] and crested larks [*Galerida cristata* (Linnaeus 1758)] were captured at various sites in Saudi Arabia, as described by Tieleman and Williams (Tieleman and Williams, 2002) and Tieleman et al. (Tieleman et al., 2003). We captured skylarks (*Alauda arvensis* Linnaeus 1758) from various locations in the Netherlands and western Germany, and horned larks [*Eremophila alpestris* (Linnaeus 1758)] near Columbus, OH. Birds were either processed for lipid analysis immediately or frozen in an atmosphere of nitrogen gas for later processing. In addition to the larks that we directly captured, we also used data from Ro (Ro, 2009) and Ro and Williams (Ro and Williams, 2010) to add 13 non-lark species for our analysis of CWL and intercellular SC lipids. Experiments were approved by IACUC at The Ohio State University (2009A0074-R1).

### Environments of bird sampling locations

To quantify environmental conditions for each species in terms of temperature and moisture availability, we calculated an aridity index for each species' environment as $Q=P/[(T_{max}+T_{min})(T_{max}-T_{min})]\times-1000$, where $P$ is the average annual precipitation (mm), $T_{max}$ is the mean maximum temperature of the hottest month (°C) and $T_{min}$ is the mean minimum temperature of the coldest month (°C) (Emberger, 1955). $Q$ is low in hot, dry deserts and high in cool, wet areas. We collected climatic data from the literature (Tieleman et al., 2003; Walter and Lieth, 1967; Williams, 2001), and from www.worldclimate.com and www.onlineweather.com. Because $Q$ increases rapidly when environments become more mesic, we avoided unequal weighting of data for mesic species by using log$Q$ in our analyses (Tieleman et al., 2003).

### CWL

For every lark species except the horned lark, we did not measure CWL for the same individuals as used for lipid analysis. Therefore, we used mean values of CWL measured at 25°C for skylarks, hoopoe larks, Dunn's larks and desert larks from data in Tieleman and Williams (Williams, 2002) and Haugen et al. (Haugen et al., 2003). We measured CWL of horned larks with a standard flow-through respirometry method identical to the methods used to measure the larks above, except that we measured horned larks at 30°C. Measurements of desert and mesic lark species have shown no differences in CWL between 25 and 30°C (Tieleman and Williams, 2002). For the 13 non-lark species, we used mean values of CWL measured at 30°C. For all non-lark species, the individuals were the same for CWL and lipid analysis (Ro and Williams, 2010; Ro, 2009). In addition to the direct measurements of CWL for the species listed above, we used estimates of CWL values for black-crowned finchlarks and crested larks from Haugen et al. (Haugen et al., 2003).

### Extraction of intercellular lipids from the SC of larks

To extract lipids from the SC of larks, we plucked feathers, peeled the skin away from the body, and pinned the skin on a Teflon sheet covered with filter paper. We saturated the filter paper with a 0.5% trypsin (FisherChemical laboratory grade) solution in phosphate buffered saline (PBS), and incubated the skin at 4°C for 24–48 h to separate the epidermis from the dermis. After this period, the epidermis was immersed in a fresh 0.5% trypsin solution in PBS and incubated for 3 h at 37°C to isolate the SC from the rest of the epidermis. The trypsin solution was rinsed with distilled water, and the SC was placed in a 10 ml vial and freeze-dried overnight to extract water. Thereafter, we determined the dry mass of the SC, and extracted the intercellular lipids by placing the SC in successive baths of 2:1, 1:1 and 1:2 (v/v) chloroform:methanol for 2 h each. Each bath also contained 50 mg $l^{-1}$ of the antioxidant butylated hydroxytoluene (BHT). We combined the extracts and evaporated the solvent with a stream of nitrogen in a nitrogen manifold (N-EVAP, model 11155-O, Organomation Associates, Berlin, MA, USA). We then re-constituted the lipids in enough 2:1 chloroform:methanol with 50 mg $l^{-1}$ BHT to fully dissolve the sample (~100–300 µl).

### Extraction of CBLs from the SC of larks

To confirm that all intercellular lipids of the SC were extracted, we re-soaked the SC for each bird for 2 h in 1:2 (v/v) chloroform:methanol, and examined extracts for lipids with thin

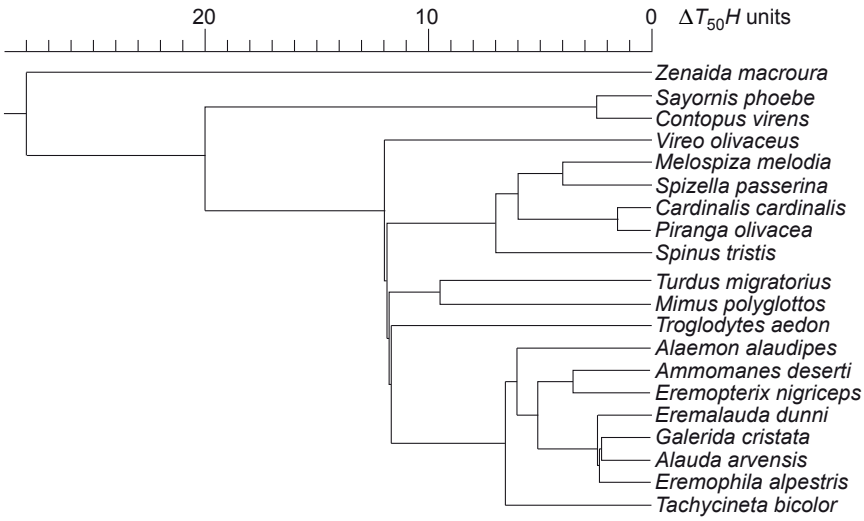


Fig. 1. Phylogenetic tree of all species in this study. $\Delta T_{50}H$ represents units of difference in melting temperatures of bonded DNA strands for different species.

layer chromatography (TLC). No lipid bands were detected in our plates, indicating that all the intercellular lipids had been removed. We then extracted CBLs by immersing the SC in 2 ml of 1 mol $l^{-1}$ NaOH in 90% methanol at 60°C for 2 h (Wertz and Downing, 1987). This mild alkaline hydrolysis breaks the ester bonds between lipids and proteins (Wertz and Downing, 1987). We then adjusted the pH to 6 by adding 3 mol $l^{-1}$ HCl, and added 2.5 ml of chloroform. The solution was then passed through a sintered glass filter and centrifuged at 3000 ***g*** for 15 min. After a few minutes, the solution separated into two layers, an aqueous layer and an organic layer that contained lipids. The organic phase was washed twice with distilled water to remove contaminants. The aqueous phase was mixed with 1 ml of chloroform to extract any lipids that might be in this phase, and centrifuged again at 3000 ***g*** for 10 min. We combined the organic fractions, and removed any remaining small particles by passing the solution through a PTFE filter, 0.45 µm pore size (Millex, Millipore, Bedford, MA, USA). This method has been verified through TLC of the aqueous fraction to extract all lipids to the organic fraction (E. A. Calhoon and J. B. Williams, unpublished). We dried the filtrate with a stream of nitrogen and re-constituted the sample in enough chloroform:methanol (2:1 v/v) with 50 mg $l^{-1}$ of BHT to fully dissolve the sample (30–170 µl).

### Quantification of intercellular lipids and CBLs

We used TLC to analyze the amounts of lipid classes in the SC of larks. For our analysis, we used 20×20 cm glass plates coated with 0.25-mm-thick silicic acid (Adsorbosil-Plus 1, Altech, Deerfield, IL, USA). A 2:1 $CHCl_3$:MeOH solution run to the top of the plates removed contaminants prior to sample loading. Following this washing, we activated the plates by heating them in an oven for 30 min at 110°C, and then scored the silicic acid to create 13 separate lanes on each plate. For each sample, we ran one plate to detect relatively polar lipids, such as cerebrosides, ceramides and cholesterol, and a separate plate to detect non-polar lipids, such as free fatty acids, triacylglycerides, methyl esters and cholesterol esters. On the polar plate, we grouped ceramides into three categories, from ceramide I, comprising the least polar classes, through ceramide III, comprising the most polar classes, because the polarity of ceramides has been shown to be important in CWL (Muñoz-Garcia et al., 2008). We used a Hamilton syringe with a Teflon-coated tip to load a set of five standards comprised of each lipid class dissolved in 2:1 chloroform:methanol at known concentrations on each plate. Our standard was serially diluted relative to the most concentrated standard to produce a range of lipid concentrations with which we compared the samples. After loading the standards, we loaded each sample into two lanes and then developed the plates. To separate polar lipids, we developed the plate with 60:40:5 chloroform:methanol:water run 10 cm from the bottom, followed by 190:9:1 chloroform:methanol:acetic acid run 15 cm from the bottom and finally 70:30:1 hexane:ethyl ether:acetic acid run to the top. We developed nonpolar plates with 80:20:2 hexane:ethyl ether:acetic acid run to the top. After developing the plates, we allowed them to dry, sprayed them with a solution of 3% cupric acetate in 8% phosphoric acid and heated them in an oven for 30 min at 180°C. This procedure chars the lipids to allow visualization. We scanned the plates on a Hewlett-Packard (Scanjet 5590) scanner and quantified each lipid class with IMAL (Nelson, 2003). For the analysis of intercellular lipids in non-larks, we used data from Ro (Ro, 2009) and Ro and Williams (Ro and Williams, 2010), in which the procedure for lipid extraction and TLC was identical to ours.

### Statistics

We performed all statistical tests with SPSS 19.0 (IBM, Armonk, NY, USA), with statistical significance set at $P<0.05$. We tested for correlations between mean lipid class amounts or percentages and

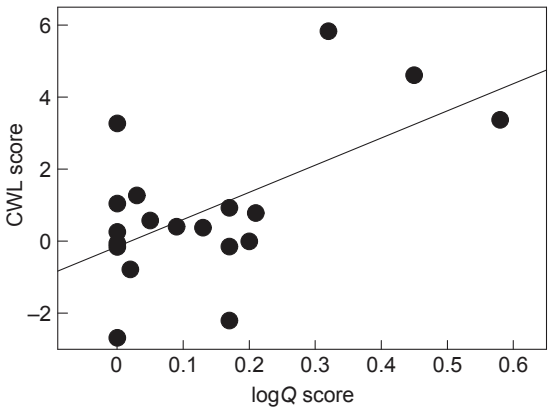


Fig. 2. Phylogenetically independent contrast scores for average cutaneous water loss (CWL) for 20 species of birds (19 contrasts) *versus* the aridity index (log$Q$). Values are made positive on the *x*-axis.

Table 1. Cutaneous water loss (mg cm$^{-2}$ day$^{-1}$) and intercellular lipid values for all species of birds (mg g$^{-1}$ stratum corneum) in the present study

| Common name (*N*) | Latin name | CWL (mg cm$^{-2}$ day$^{-1}$) | Cholesterol ester | FAME | TAG | FFA | Cholesterol | Ceramide I | Ceramide II | Ceramide III | Cerebroside |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Black-crowned finchlark (8) | *Eremopterix nigriceps* | 14.0 | 25.1±3.5 | 8.7±1.0 | 26.5±3.4 | 36.8±4.9 | 8.8±0.8 | 5.7±0.7 | 8.5±1.1 | 32.6±3.2 | 78.4±11.1 |
| Dunn's lark (7) | *Eremalauda dunni* | 14.5 | 20.0±5.0 | 7.3±1.2 | 17.6±2.5 | 26.2±3.2 | 6.3±1.0 | 3.9±0.5 | 7.5±0.7 | 19.7±3.8 | 52.9±10.8 |
| Crested lark (9) | *Galerida cristata* | 15.8 | 19.0±2.3 | 10.1±1.3 | 33.2±4.2 | 47.1±8.6 | 7.1±0.5 | 4.4±0.4 | 8.2±0.6 | 22.4±2.7 | 55.7±5.7 |
| Hoopoe lark (9) | *Alaemon alaudipes* | 18.5 | 18.4±3.6 | 8.0±1.2 | 21.3±2.3 | 23.0±4.2 | 5.9±0.6 | 3.3±0.5 | 7.4±1.3 | 20.6±3.3 | 49.5±8.4 |
| Desert lark (6) | *Ammomanes deserti* | 20.0 | 19.1±1.9 | 16.0±2.3 | 26.3±3.9 | 36.6±9.1 | 10.6±2.2 | 4.6±0.5 | 8.6±1.3 | 25.1±2.3 | 60.5±3.8 |
| Horned lark (11) | *Eremophila alpestris* | 20.8±1.4 | 8.8±1.0 | 168.7±31.7 | 19.0±5.0 | 13.8±1.7 | 8.4±0.5 | 5.7±0.4 | 2.7±0.7 | 44.3±5.7 | 42.7±5.3 |
| Chipping sparrow (5) | *Spizella passerina* | 21.8±2.3 | 34.2±7.2 | 44.9±29.9 | 41.9±11.3 | 8.2±3.6 | 3.0±1.3 | 0 | 7.6±1.1 | 58.9±5.3 | 32.8±4.8 |
| Mourning dove (5) | *Zenaida macroura* | 22.6±3.5 | 4.4±1.7 | 46.2±17.7 | 35.4±10.7 | 38.2±26.9 | 2.1±0.6 | 0.8±0.8 | 25.0±8.6 | 25.5±5.4 | 11.6±2.5 |
| Northern cardinal (6) | *Cardinalis cardinalis* | 24.9±3.7 | 29.8±7.2 | 51.1±13.4 | 28.7±3.7 | 4.6±2.1 | 0.8±0.2 | 0 | 10.8±1.4 | 45.0±3.8 | 24.3±3.4 |
| Northern mockingbird (5) | *Mimus polyglottos* | 25.1±3.1 | 15.9±4.1 | 33.1±17.6 | 60.2±15.4 | 8.2±6.2 | 1.5±0.5 | 1.3±0.7 | 11.4±4.3 | 37.6±3.9 | 18.4±1.7 |
| Skylark (10) | *Alauda arvensis* | 25.7 | 13.2±2.1 | 10.2±1.6 | 68.7±13.0 | 67.0±7.2 | 9.1±0.6 | 5.0±0.6 | 0.0±0.0 | 19.8±2.2 | 27.9±4.8 |
| American robin (5) | *Turdus migratorius* | 25.8±3.4 | 22.8±3.7 | 10.1±3.3 | 75.6±18.1 | 6.9±4.2 | 6.0±3.5 | 0 | 16.5±3.6 | 52.1±6.8 | 26.0±6.9 |
| American goldfinch (6) | *Spinus tristis* | 26.5±3.2 | 27.3±6.0 | 41.3±16.9 | 48.2±15.8 | 26.2±12.8 | 4.0±3.4 | 0.1±0.1 | 8.6±0.8 | 63.8±3.8 | 24.4±2.8 |
| House wren (6) | *Troglodytes aedon* | 26.7±2.1 | 46.4±6.4 | 42.0± 16.9 | 22.1±3.3 | 13.9±7.3 | 1.9±0.6 | 0.2±0.2 | 9.1±1.1 | 58.0±9.1 | 37.4±4.8 |
| Scarlet tanager (4) | *Piranga olivacea* | 26.7±2.3 | 10.9±2.2 | 69.8±23.9 | 32.9±8.0 | 2.9±2.2 | 1.8±0.3 | 0 | 13.5±2.0 | 51.7±8.1 | 37.4±4.9 |
| Red-eyed vireo (6) | *Vireo olivaceus* | 27.2±2.6 | 52.1±13.2 | 123.7±58.1 | 42.2±12.2 | 21.2±15.1 | 0.4±0.1 | 0 | 13.4±2.5 | 59.5±11.8 | 30.5±4.8 |
| Eastern wood-pewee (1) | *Contopus virens* | 28.5 | 6.6 | 52.2 | 0.1 | 0.7 | 0.8 | 0 | 11.2 | 32.6 | 19.6 |
| Song sparrow (6) | *Melospiza melodia* | 29.4±2.3 | 44.2±11.7 | 53.3±14.8 | 44.3±17.8 | 18.9±9.8 | 1.5±1.2 | 0 | 9.0±1.9 | 52.0±7.4 | 23.6±4.2 |
| Tree swallow (5) | *Tacycineta bicolor* | 35.6±4.9 | 13.4±3.4 | 64.9±29.8 | 56.7±25.8 | 4.5±2.8 | 2.4±0.6 | 0 | 14.8±3.4 | 59.5±12.5 | 27.6±2.2 |
| Eastern phoebe (5) | *Sayornis phoebe* | 35.8±2.1 | 40.7±16.6 | 40.8±23.7 | 60.6±18.6 | 4.2±3.1 | 3.5±0.4 | 0.2±0.2 | 5.9±0.1 | 48.7±5.1 | 22.8±3.6 |

CWL, cutaneous water loss; FAME, fatty acid methyl esters; FFA, free fatty acids; *N*, sample size; TAG, triacylglycerides.
Data for CWL for larks, except horned lark, are taken from Tieleman and Williams (Tieleman and Williams, 2002) and Haugen (Haugen, 2003), and all data for nonlarks are taken from Ro and Williams (Ro and Williams, 2010) and Ro (Ro, 2009).
All values are means ± s.e.m., where information on s.e.m. was available.

CWL or log*Q* for each species with stepwise multiple linear regression. Percentages were logit transformed [ln(*Y*/1–*Y*)] to normalize the data.

Our data consisted of 15 mesic species and five desert species, the latter of which were all larks. Because the only desert species were larks, we calculated phylogenetic independent contrasts (PIC) of our physiological data to account for relatedness among species. We constructed our phylogenetic tree based on Sibley and Ahlquist (Sibley and Ahlquist, 1990), modified by Boyd (Boyd, 2011) (Fig. 1). Branch lengths were based on Sibley and Ahlquist (Sibley and Ahlquist, 1990). From this tree, we calculated PIC on CWL, and amounts, percentages and ratios of lipid classes, with the Phenotypic Diversity Analysis Program module of PDTREE (Garland et al., 1999; Garland and Ives, 2000).

To explore the interactions between lipid classes and their effects on CWL, we used principal component analysis (PCA) on the percent (logit transformed) of each class of lipid (Shaw, 2003). This analysis yielded uncorrelated composite variables, the principal components. We used the program 'Factor analysis' in SPSS without rotation to extract components with eigenvalues greater than one as our selection criterion. We then used linear regression to determine associations between each principal component and mean CWL for each species. We also attempted to use PCA on the amounts of each lipid class, but we did not find enough correlation between variables to justify the use of PCA (Kaiser–Meyer–Olkin measure of sampling adequacy=0.577).

Within larks, we used the same tests as described above to test for the effects of CBLs on CWL. Because all species were members of the same family, we did not calculate PIC, as previous studies on CWL in larks have shown no differences between phylogenetically informed and standard linear regression methods (Tieleman et al., 2003). We also attempted to perform PCA for CBLs in larks, but there was not enough covariance between variables to adequately reduce the number of variables. Finally, to test for similarities between the intercellular lipids and CBLs, we tested for correlations between covalently bound and intercellular lipid classes among all individual larks by calculating Pearson's correlation coefficients. Because we correlated multiple lipid classes, we applied a Bonferroni correction to account for multiple comparisons (Zar, 1996).

## RESULTS

### CWL and environment

For all 20 species of birds for which we had physiological data, we found a significant positive association between log*Q* and CWL ($R^2$=0.42, $P$=0.003; Fig. 2), demonstrating that rates of CWL in birds are higher in more mesic regions than more arid regions.

### Intercellular lipids and CWL

Among all 20 species, we found that the main lipid classes in the intercellular spaces of the SC of birds were fatty acid methyl esters, triacylglycerides, ceramide III and cerebroside, which made up over 70% of all lipids (Table 1). The amount of triacylglycerides correlated positively with CWL, whereas the amount of ceramide I correlated negatively with CWL ($R^2$=0.758, $P$<0.001 and 0.044, respectively; Fig. 3). The percentage of cerebrosides was negatively correlated with CWL ($R^2$=0.61, $P$<0.001; Fig. 4).

When we correlated ratios of lipid classes with CWL, the ratio of ceramides to cerebrosides correlated positively with CWL ($R^2$=0.47, $P$=0.001; Fig. 4), providing evidence that cerebrosides may decrease CWL.

In a PCA analysis of the percentage of each lipid class relative to total lipids, two axes accounted for 71.3% of the variance. A

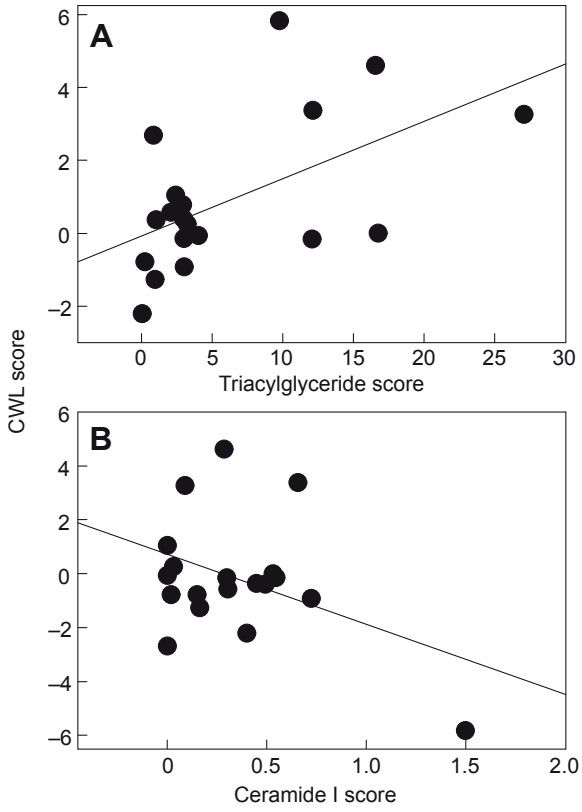


Fig. 3. Phylogenetically independent contrast scores for average (A) triacylglycerides and (B) ceramide I *versus* average cutaneous water loss (CWL) in 20 species of birds. Values are made positive on the *x*-axis.

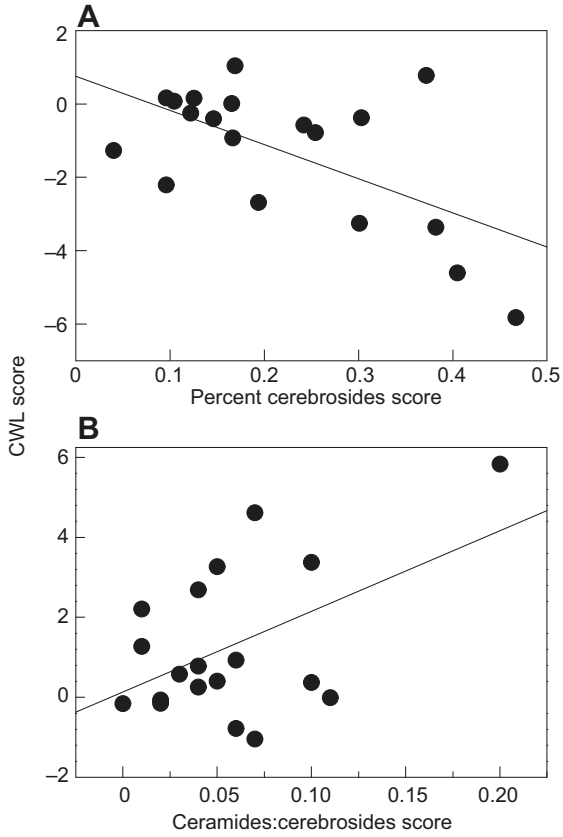


Fig. 4. Phylogenetically corrected (A) percent cerebrosides and (B) ratio of ceramides to cerebrosides *versus* cutaneous water loss (CWL). Values are made positive on the *x*-axis.

plot of scores for each species along these axes revealed that principal component 1 (PC1) separated desert species from mesic species (Fig. 5A). Stepwise regression analysis of both principal components against CWL demonstrated that PC1 was negatively correlated with CWL ($R^2$=0.57, $P$<0.001). Furthermore, a plot of the eigenvector loadings of each lipid class (Fig. 5B) showed a separation of lipid classes into three distinct groups. PC1 separated free fatty acids, cholesterol, ceramide I and cerebroside from fatty acid methyl esters, ceramide II and ceramide III. Principal component 2 (PC2) separated these lipid classes from triacylglycerides. Combining the species plot with the lipid plot suggests that a combination of free fatty acids, cholesterol, ceramide I and cerebroside was associated with desert species, whereas a combination of fatty acid methyl esters, ceramide II and ceramide III was more associated with mesic species. Because the scores for most desert birds and the score for triacylglycerides were negative along PC2, it is also possible that the modification of triacylglycerides is important for desert birds.

Although we could find no clear pattern in polarity or molecular structure between the groups of lipid classes separated by PC1 and PC2, the interactions between these lipid classes may provide insight into the regulation of CWL by the SC. Therefore, we tested for an association between each principal component and CWL. In a stepwise regression analysis, we found that PC1 was negatively associated with CWL ($R^2$=0.64, $P$<0.001).

### CBLs of larks

Within larks, we found that the main classes of CBLs in the SC were cerebrosides, three classes of ceramides, cholesterol, free fatty acids, triacylglycerides, fatty acid methyl esters and cholesterol esters (Table 2). Together, free fatty acids, cerebrosides and ceramides accounted for over 80% of all CBLs. We found a positive correlation between the amount and percentage of triacylglycerides and CWL ($R^2$=0.76, $P$=0.047 and $R^2$=0.76, $P$=0.048, respectively), despite the fact that triacylglycerides made up less than 2% of all CBLs. When we performed multiple regression analysis on how amounts of CBLs in larks correlated with log$Q$, we found that ceramide III correlated negatively with log$Q$, whereas cholesterol correlated positively with log$Q$ ($R^2$=0.327, $P$<0.001, $P$=0.004, respectively).

### Correlations between intercellular lipids and CBLs of the SC of larks

Within larks, we found significant positive correlations between the amount of intercellular and covalently bound cerebrosides ($R^2$=0.21, $P$=0.001), intercellular cholesterol ester and covalently bound cerebrosides ($R^2$=0.23, $P$<0.001) and intercellular cerebrosides and covalently bound free fatty acids ($R^2$=0.22, $P$<0.001; Fig. 6).

## DISCUSSION

We found that birds from arid environments had lower rates of CWL than birds from more mesic environments, and a PCA analysis revealed that these lower rates of water loss may be associated with interactions between intercellular free fatty acids, cholesterol, ceramide I and cerebroside. This potential interaction is difficult to interpret, because there were no clear patterns of polarity or molecular structure within this group compared with lipids associated with higher rates of CWL.

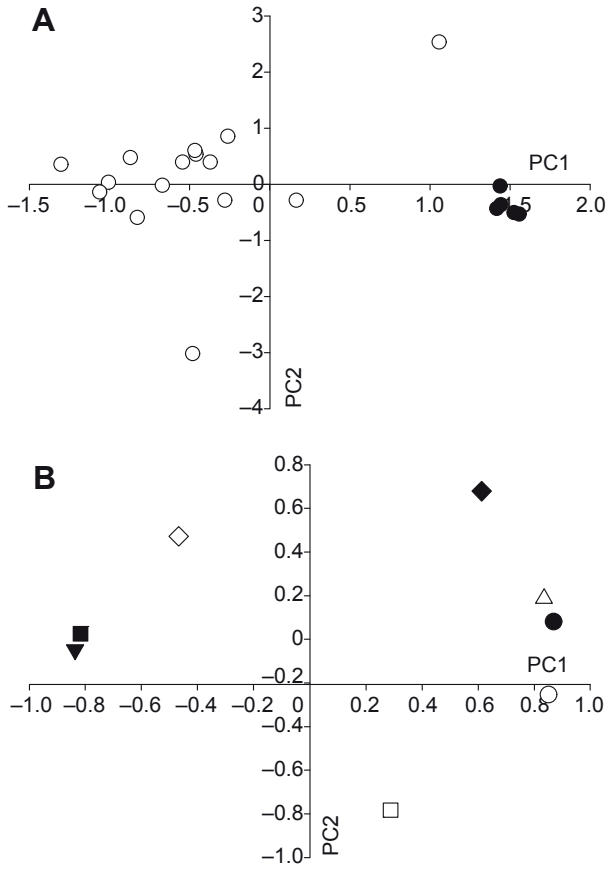


Fig. 5. (A) Principal component scores for five desert birds (filled circles) and 15 mesic birds (unfilled circles). (B) Eigenvector loadings for cerebroside (filled diamond), ceramide III (filled inverted triangle), ceramide II (unfilled diamond), ceramide I (filled circle), cholesterol (unfilled triangle), free fatty acids (unfilled circle), triacylglycerides (unfilled square) and fatty acid methyl esters (filled square).

One pattern that emerged in both PCA and linear regression analyses was a consistent negative association between cerebrosides and CWL. This association is counterintuitive, as the sugar moieties of the cerebrosides are bulky and potentially bind with water to disrupt lipid packing and increase CWL (Rawlings and Matts, 2005). Furthermore, mice with Gaucher disease, with ~7 μg of intercellular cerebrosides per $cm^2$ of SC, have an average CWL rate of 9.8 mg $cm^{-2}$ $day^{-1}$, whereas controls average only 0.24 mg $cm^{-2}$ $day^{-1}$ (Holleran et al., 1994). Using Meeh's equation (Meeh, 1879), we calculated skin surface area for all species in this study based on body mass, and thus were able to calculate intercellular cerebrosides in units of μg $cm^{-2}$ SC. We found that birds averaged ~14 μg cerebrosides $cm^{-2}$ SC, approximately twice as much as mice with Gaucher disease. Despite this large discrepancy, this study and others have found that an increase in cerebrosides is associated with lower CWL in birds (Muñoz-Garcia and Williams, 2005; Gu et al., 2008). These studies taken together suggest that in avian SC, cerebrosides are arranged in a way that prevents, rather than facilitates, water loss, a pattern opposite to that found in mammals.

The current model for intercellular organization in avian SC is a modification of the sandwich model proposed by Bouwstra (Bouwstra et al., 2000) for mammalian systems (Muñoz-Garcia et al., 2008). In this model, cerebrosides are arranged in the center of two outer layers of ceramides to form a trilayer of lipids. The hexose moieties of the cerebrosides therefore interdigitate with the lipid tail groups of ceramides and free fatty acids (Fig. 7A). However, under this model, the hexose moieties would potentially attract water molecules inside the lipid layers, where they may disrupt lipid packing, and increase CWL (Golden et al., 1986). Because our data suggest that an increase in intercellular cerebrosides decreases water loss through the SC, we suggest that lipids are arranged in the avian SC in a bilayer, rather than in trilayers as previously envisioned. We hypothesize that lipid molecules are arranged within each layer with hydrophilic headgroups facing outward and hydrophobic tails facing inward. We argue that the sugar moieties of the cerebrosides are located on the outside of the lipid layers, where they could interact with water molecules without disrupting lipid packing (Fig. 7B). Each cerebroside molecule interacts with four to nine water molecules (Bach et al., 1982), and these water molecules can influence the hydrogen bonding of surrounding water molecules to form solvation shells. In this way, the cerebrosides could potentially sequester water molecules within the SC by ordering them around the hydroxyl groups of the hexose moieties. This ordered water would potentially form aggregates in which water molecules exhibit strong hydrogen bonding. These hydrogen bonds would raise the energy required for each water molecule to percolate through the SC, thus lowering CWL (Clement et al., 2012). Hydrogen bond strength of water and lipid packing can be evaluated by infrared spectroscopy (Golden et al., 1986; Du et al., 1993). Thus, if the cerebrosides lie outside lipid bilayers, the strength of hydrogen binding in water molecules should increase as cerebroside content increases, and lipid packing will be unaffected by the addition of water into the SC. However, if cerebrosides lie inside lipid trilayers, hydrogen binding strength will remain high, but the addition of water to the SC will disrupt lipid packing as the water molecules associate with cerebrosides and consequently interact with lipid tail groups (Williams et al., 2012).

CBLs add a layer of complexity to current models of lipid organization in the avian SC (Clement et al., 2012). In current thinking, covalently bound ceramides and cerebrosides align with

Table 2. Covalently bound lipid amounts for all lark species (mg $g^{-1}$ stratum corneum)

| Species (*N*) | Cholesterol ester | FAME | TAG | FFA | Cholesterol | Ceramide I | Ceramide II | Ceramide III | Cerebroside |
|---|---|---|---|---|---|---|---|---|---|
| Black-crowned finchlark (7) | 0.4±0.4 | 2.1±0.4 | 0 | 15.6±2.3 | 0.5±0.2 | 0.4±0.2 | 0.1±0.1 | 3.4±0.9 | 5.9±1.6 |
| Dunn's lark (7) | 0.3±0.3 | 2.0±0.4 | 0 | 11.4±1.8 | 0.1±0.1 | 0.9±0.2 | 0 | 3.0±0.7 | 3.6±0.6 |
| Crested lark (7) | 0.9±0.4 | 3.3±0.8 | 0.1±0.1 | 10.1±1.6 | 0.4±0.1 | 0.3±0.2 | 0 | 3.6±1.6 | 4.6±0.8 |
| Hoopoe lark (8) | 0.3±0.1 | 4.8±1.5 | 0.1±0.1 | 7.9±1.7 | 0.1±0.1 | 0.3±0.1 | 0.1±0.1 | 7.4±2.6 | 4.4±0.6 |
| Desert lark (6) | 3.6±1.7 | 2.9±0.3 | 0.2±0.2 | 16.9±1.5 | 0.7±0.3 | 0.4±0.2 | 0 | 8.5±1.8 | 4.7±1.0 |
| Horned lark (9) | 0.1±0.1 | 1.1±0.2 | 0.3±0.1 | 8.8±2.1 | 0.6±0.2 | 0.7±0.1 | 0 | 1.3±0.1 | 3.8±0.6 |
| Skylark (9) | 0.1±0.1 | 1.3±0.2 | 0.2±0.1 | 8.7±1.5 | 0.5±0.1 | 0.6±0.1 | 0.1±0.1 | 1.5±0.2 | 3.3±0.6 |

FAME, fatty acid methyl esters; FFA, free fatty acids; *N*, sample size; TAG, triacylglycerides.
All values are means ± s.e.m.

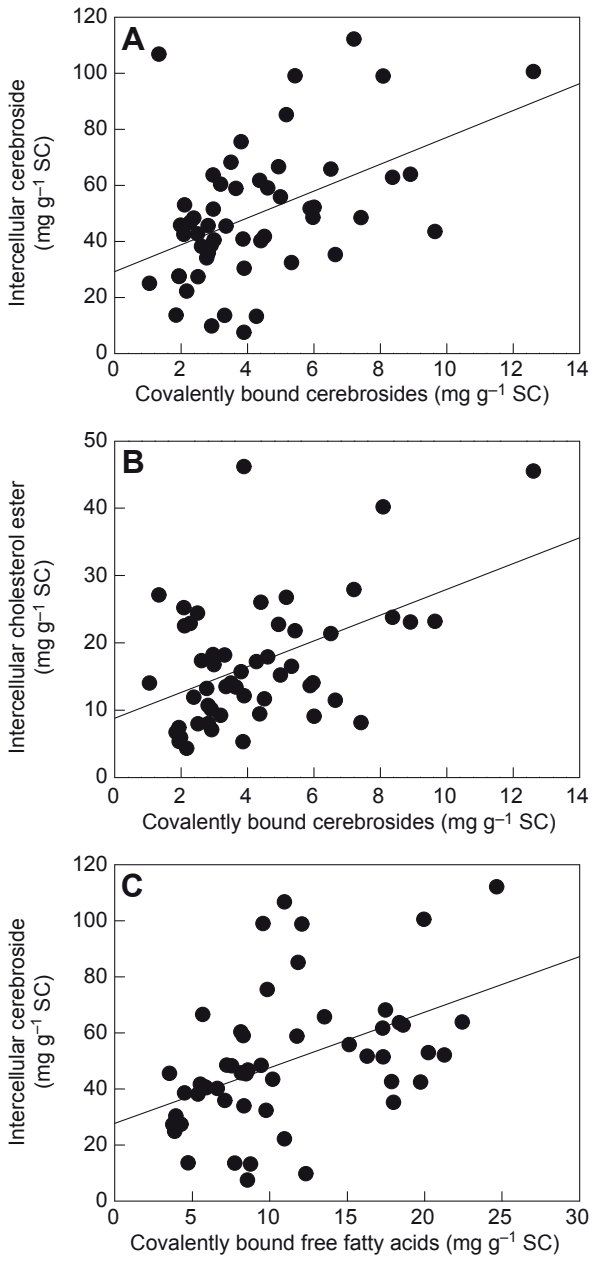


Fig. 6. Correlations of covalently bound lipids with intercellular lipids for individual larks. (A) Covalently bound cerebrocides *versus* intercellular cerebrosides, (B) covalently bound cerebrosides *versus* intercellular cholesterol esters and (C) covalently bound free fatty acids *versus* intercellular cerebrosides.

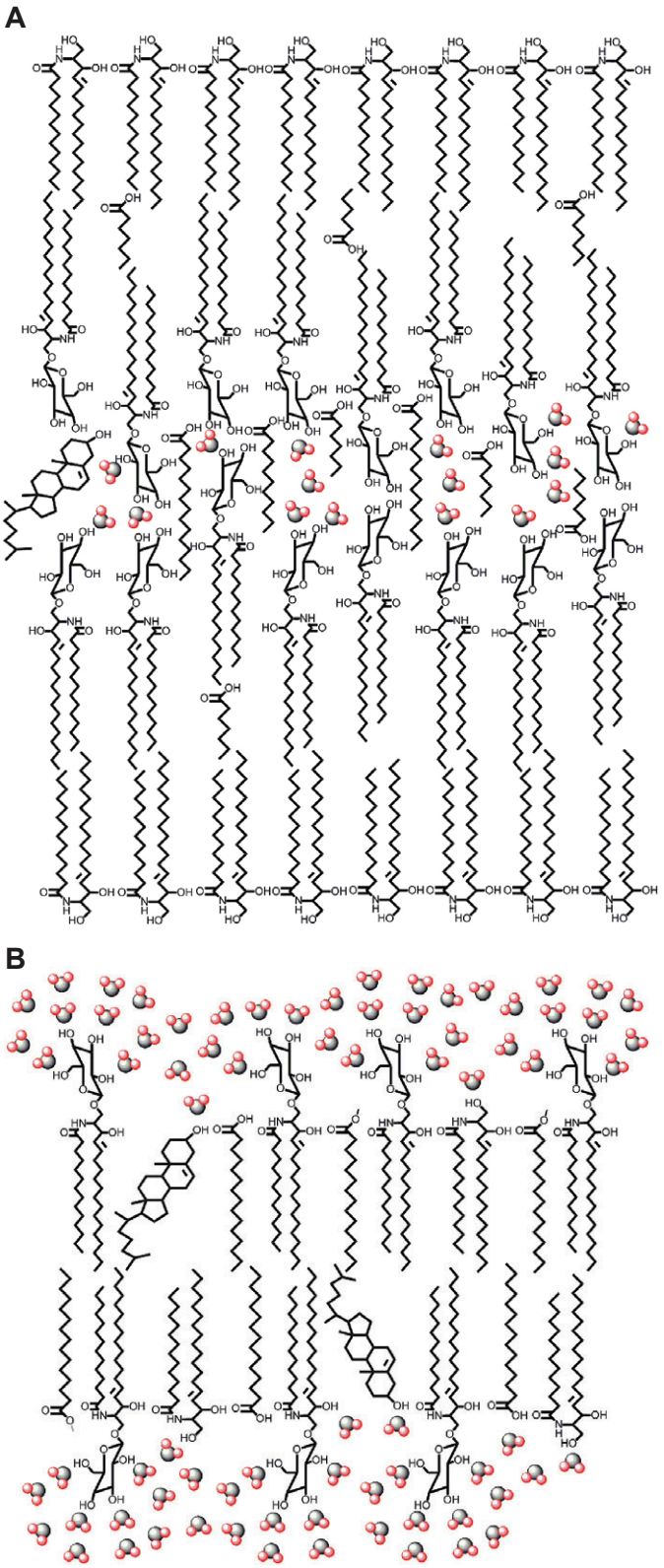


Fig. 7. (A) The sandwich model for intercellular lipids in the avian stratum corneum. Adapted from Muñoz-Garcia et al. (Muñoz-Garcia et al., 2005). (B) The bilayer model for intercellular lipids in the avian stratum corneum.

their polar headgroups facing away from the corneocytes, where the cerebrosides may interact with water molecules. Shorter-chained, less polar lipids such as free fatty acids and cholesterol esters occupy the remaining binding sites on the corneocytes to create a CBL layer that inhibits water permeation through the corneocytes, and potentially interacts with the nearest intercellular lipid bilayer to create a water shell between the covalently bound and intercellular lipid layers.

Models of mammalian SC have suggested that the covalently bound and intercellular lipids interdigitate at their respective head groups to create links between adjacent corneocytes (Wertz and Downing, 1987). However, if cerebrosides are packed too tightly together, the lamellar structure breaks down (Maggio et al., 2006). Therefore, if intercellular lipids and CBLs interdigitate in avian SC, the cerebrosides must be spaced far enough apart to prevent

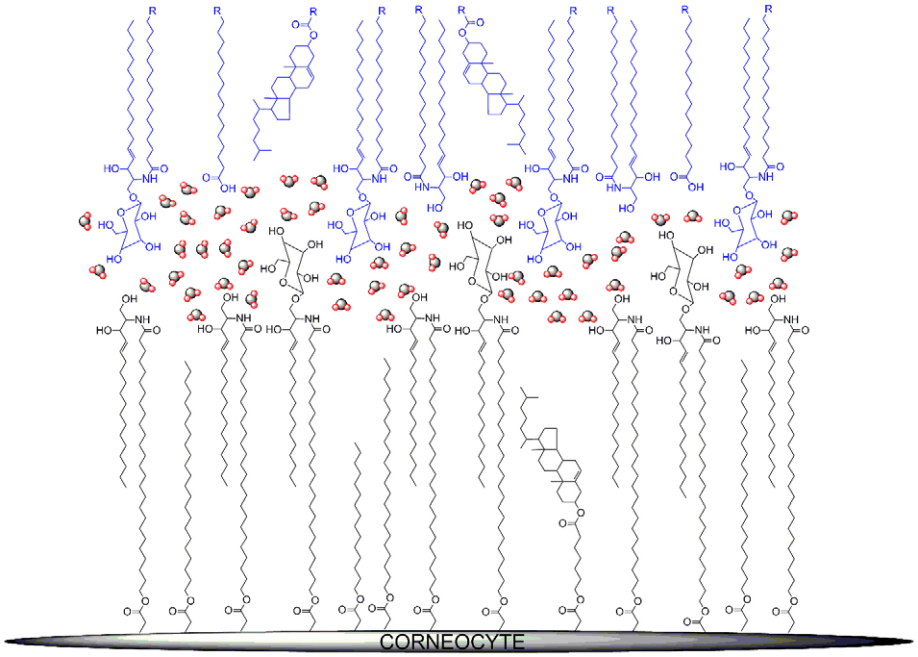


Fig. 8. Model for the interface of covalently bound and intercellular lipids within the avian stratum corneum, showing interdigitation between intercellular and covalently bound cerebrosides. Covalently bound lipids are colored black, and intercellular lipids are colored blue.

disruptions of the lamellar structure. Although we found a positive correlation between intercellular and covalently bound cerebrosides, we also found that as the amount of intercellular cerebroside increased, covalently bound free fatty acids also increased, and as covalently bound cerebrosides increased, intercellular cholesterol ester increased. These correlations suggest that as the amount of cerebroside increases in either the covalently bound or intercellular lipid layer, the opposing layer incorporates more short-chained, less polar lipids to increase spacing between cerebroside molecules. This increase in spacing may allow the lipid layers to interdigitate even when high amounts of cerebrosides are present (Fig. 8).

In conclusion, we found that birds in arid environments have lower rates of CWL than birds from mesic environments. These differences in water loss may be explained by differences in the interactions between and among intercellular lipids and CBLs. Although these interactions are unclear, a consistent pattern that has emerged is the negative correlation between cerebrosides and CWL. This finding represents an enigma given how cerebrosides affect CWL in mammals (Holleran et al., 1994; Rawlings and Matts, 2005), which has prompted us to evaluate a new model for the organization of lipids in the avian SC. In this model, cerebrosides are arranged in a way that potentially prevents, rather than facilitates, water loss. While this model is speculative, tests on this model using infrared spectroscopy (Williams et al., 2012) may allow us to gain a better understanding of how lipids interact within the SC. Such an understanding will allow us to make better predictions of how birds change their physiology in response to environmental change. As the climate becomes hotter and drier in many regions (IPCC, 2007), birds may increase cerebroside content in their SC, either through natural selection or phenotypic plasticity.

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| BHT | butylated hydroxytoluene |
| CBL | covalently bound lipid |
| CWL | cutaneous water loss |
| PBS | phosphate buffered saline |
| PCA | principal component analysis |
| PIC | phylogenetic independent contrast |
| *Q* | aridity index |
| SC | stratum corneum |
| TLC | thin layer chromatography |

## ACKNOWLEDGEMENTS

We would like to thank members of the Williams laboratory – Liz Calhoon, Clara Cooper-Mullin, Ana Jimenez, Andrew Sudimack and James VanBrocklyn – for their helpful comments on earlier drafts of this manuscript. We also thank all private landowners for allowing us to capture birds on their property.

## FUNDING

Funding for this project was supplied by the National Science Foundation [IBW-0212092 to J.B.W.].

## REFERENCES

**Bach, D., Sela, B. and Miller, I. R.** (1982). Compositional aspects of lipid hydration. *Chem. Phys. Lipids* **31**, 381-394.
**Bartholomew, G. A.** (1972). The water economy of seed-eating birds that survive without drinking. *Proc. Int. Ornithol. Congr.* **15**, 1-16.
**Bouwstra, J. A.** (1997). The skin barrier, a well-organized membrane. *Colloids Surfaces A* **123**, 403-413.
**Bouwstra, J. A., Dubbelaar, F. E. R., Gooris, G. S. and Ponec, M.** (2000). The lipid organization in the skin barrier. *Acta Derm. Venereol.* **208**, 23-30.
**Boyd, J. H., III** (2011). Aves – a taxonomy in flux. Version 2.5. Available at http://jboyd.net/Taxo/taxo1.html.
**Clement, M. E., Muñoz-Garcia, A. and Williams, J. B.** (2012). Cutaneous water loss and covalently bound lipids of the stratum corneum in nestling house sparrows (*Passer domesticus* L.) from desert and mesic habitats. *J. Exp. Biol.* **215**, 1170-1177.
**Cox, R. M., Munoz-Garcia, A., Jurkowitz, M. S. and Williams, J. B.** (2008). β-Glucocerebrosidase activity in the stratum corneum of house sparrows following acclimation to high and low humidity. *Physiol. Biochem. Zool.* **81**, 97-105.
**Dawson, W. R.** (1982). Evaporative losses of water by birds. *Comp. Biochem. Physiol.* **71A**, 495-509.
**Downing, D. T.** (1992). Lipid and protein structures in the permeability barrier of mammalian epidermis. *J. Lipid Res.* **33**, 301-313.
**Du, Q., Superfine, R., Freysz, E. and Shen, Y. R.** (1993). Vibrational spectroscopy of water at the vapor/water interface. *Phys. Rev. Lett.* **70**, 2313-2316.
**Emberger, L.** (1955). Afrique du Nord-Ouest. In *Plant Ecology: Reviews of Research*, pp. 219-249. Paris, France: UNESCO.
**Garland, T., Jr and Ives, A. R.** (2000). Using the past to predict the present: confidence intervals for regression equations in phylogenetic comparative methods. *Am. Nat.* **155**, 346-364.
**Garland, T., Midford, P. E. and Ives, A. R.** (1999). An introduction to phylogenetically based statistical methods, with a new method for confidence intervals on ancestral values. *Am. Zool.* **39**, 374-388.
**Golden, G. M., Guzek, D. B., Harris, R. R., McKie, J. E. and Potts, R. O.** (1986). Lipid thermotropic transitions in human stratum corneum. *J. Invest. Dermatol.* **86**, 255-259.
**Gu, Y., Muñoz-Garcia, A., Brown, J. C., Ro, J. and Williams, J. B.** (2008). Cutaneous water loss and sphingolipids covalently bound to corneocytes in the

stratum corneum of house sparrows *Passer domesticus*. *J. Exp. Biol.* **211**, 1690-1695.
**Haugen, M., Williams, J. B., Wertz, P. and Tieleman, B. I.** (2003). Lipids of the stratum corneum vary with cutaneous water loss among larks along a temperature-moisture gradient. *Physiol. Biochem. Zool.* **76**, 907-917.
**Holleran, W. M., Ginns, E. I., Menon, G. K., Grundmann, J. U., Fartasch, M., McKinney, C. E., Elias, P. M. and Sidransky, E.** (1994). Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease. *J. Clin. Invest.* **93**, 1756-1764.
**IPCC** (2007). Climate change 2007: the physical science basis. *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (ed. S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller). Cambridge: Cambridge University Press.
**Koch, P. J., Roop, D. R. and Zhou, Z.** (2005). Cornified envelope and corneocyte-lipid envelope. In *Skin Barrier* (ed. P. M. Elias and K. R. Feingold), pp. 97-106. New York: Taylor and Francis.
**Lillywhite, H. B.** (2006). Water relations of tetrapod integument. *J. Exp. Biol.* **209**, 202-226.
**Maggio, B., Fanani, M. L., Rosetti, C. M. and Wilke, N.** (2006). Biophysics of sphingolipids II. Glycosphingolipids: an assortment of multiple structural information transducers at the membrane surface. *Biochim. Biophys. Acta* **1758**, 1922-1944.
**Meeh, K.** (1879). Oberflächenmessungen des menschlichen Körpers. *Z. Biol.* **15**, 426-458.
**Menon, G. K., Brown, B. E. and Elias, P. M.** (1986). Avian epidermal differentiation: role of lipids in permeability barrier formation. *Tissue Cell* **18**, 71-82.
**Menon, G. K., Feingold, K. R. and Elias, P. M.** (1992). Lamellar body secretory response to barrier disruption. *J. Invest. Dermatol.* **98**, 279-289.
**Meuwissen, M. E., Janssen, J., Cullander, C., Junginger, H. E. and Bouwstra, J. A.** (1998). A cross-section device to improve visualization of fluorescent probe penetration into the skin by confocal laser scanning microscopy. *Pharm. Res.* **15**, 352-356.
**Muñoz-Garcia, A. and Williams, J. B.** (2005). Cutaneous water loss and lipids of the stratum corneum in house sparrows *Passer domesticus* from arid and mesic environments. *J. Exp. Biol.* **208**, 3689-3700.
**Muñoz-Garcia, A., Ro, J., Brown, J. C. and Williams, J. B.** (2006). Identification of complex mixtures of sphingolipids in the stratum corneum by reversed-phase high-performance liquid chromatography and atmospheric pressure photospray ionization mass spectrometry. *J. Chromatogr. A* **1133**, 58-68.
**Muñoz-Garcia, A., Cox, R. M. and Williams, J. B.** (2008). Phenotypic flexibility in cutaneous water loss and lipids of the stratum corneum in house sparrows (*Passer domesticus*) following acclimation to high and low humidity. *Physiol. Biochem. Zool.* **81**, 87-96.
**Nelson, T. J.** (2003). IMAL. Available at http://www.randombio.com/imal.html.
**Rawlings, A. V. and Matts, P. J.** (2005). Stratum corneum moisturization at the molecular level: an update in relation to the dry skin cycle. *J. Invest. Dermatol.* **124**, 1099-1110.
**Ro, J.** (2009). Cutaneous and respiratory water losses of temperate birds. MSc thesis, The Ohio State University, Columbus, OH.
**Ro, J. and Williams, J. B.** (2010). Respiratory and cutaneous water loss of temperate-zone passerine birds. *Comp. Biochem. Physiol.* **156A**, 237-246.
**Schaefer, H. and Redelmeier, T. E.** (1996). Composition and structure of the stratum corneum. In *Skin Barrier. Principles of Percutaneous Absorption*, pp. 43-86. Basel, Switzerland: Karger.
**Shaw, P. J. A.** (2003). *Multivariate Statistics for the Environmental Sciences*. London: Arnold.
**Sibley, C. G. and Ahlquist, J. E.** (1990). *Phylogeny and Classification of Birds*. New Haven, CT: Yale University Press.
**Simonetti, O., Hoogstraate, A. J., Bialik, W., Kempenaar, J. A., Schrijvers, A. H., Boddé, H. E. and Ponec, M.** (1995). Visualization of diffusion pathways across the stratum corneum of native and in-vitro-reconstructed epidermis by confocal laser scanning microscopy. *Arch. Dermatol. Res.* **287**, 465-473.
**Stewart, M. E. and Downing, D. T.** (2001). The omega-hydroxyceramides of pig epidermis are attached to corneocytes solely through omega-hydroxyl groups. *J. Lipid Res.* **42**, 1105-1110.
**Swartzendruber, D. C., Wertz, P. W., Madison, K. C. and Downing, D. T.** (1987). Evidence that the corneocyte has a chemically bound lipid envelope. *J. Invest. Dermatol.* **88**, 709-713.
**Tieleman, B. I. and Williams, J. B.** (2002). Cutaneous and respiratory water loss in larks from arid and mesic environments. *Physiol. Biochem. Zool.* **75**, 590-599.
**Tieleman, B. I., Williams, J. B. and Bloomer, P.** (2003). Adaptation of metabolism and evaporative water loss along an aridity gradient. *Proc. Biol. Sci.* **270**, 207-214.
**Walter, H. and Lieth, H.** (1967) *Klimadiagramm – Weltatlas*. Jena, Germany: VEB Gustav Fischer.
**Wertz, P. W.** (2005). Biochemistry of human stratum corneum lipids. In *Skin Barrier* (ed. P. M. Elias and K. R. Feingold). New York: Taylor and Francis.
**Wertz, P. W. and Downing, D. T.** (1982). Glycolipids in mammalian epidermis: structure and function in the water barrier. *Science* **217**, 1261-1262.
**Wertz, P. W. and Downing, D. T.** (1987). Covalently bound omega-hydroxyacylsphingosine in the stratum corneum. *Biochim. Biophys. Acta* **917**, 108-111.
**Wertz, P. W., Swartzendruber, D. C., Kitko, D. J., Madison, K. C. and Downing, D. T.** (1989). The role of the corneocyte lipid envelopes in cohesion of the stratum corneum. *J. Invest. Dermatol.* **93**, 169-172.
**Williams, J. B.** (2001). Energy expenditure and water flux of freeliving dune larks in the Namib Desert: a test of the re-allocation hypothesis. *Funct. Ecol.* **15**, 175-185.
**Williams, J. B. and Tieleman, B. I.** (2000). Flexibility in basal metabolic rate and evaporative water loss among hoopoe larks exposed to different environmental temperatures. *J. Exp. Biol.* **203**, 3153-3159.
**Williams, J. B. and Tieleman, B. I.** (2005). Physiological adaptation in desert birds. *Bioscience* **55**, 416-425.
**Williams, J. B., Muñoz-Garcia, A. and Champagne, A.** (2012). Climate change and cutaneous water loss of birds. *J. Exp. Biol.* **215**, 1053-1060.
**Zar, J. H.** (1996). *Biostatistical Analysis*. Englewood Cliffs, NJ: Prentice Hall.